Applied and Environmental Microbiology, May 1999, p. 1871–1875 Vol. 65, No. 5
0099-2240/99/$04.00+0

Differentiation of *Vibrio alginolyticus* Strains Isolated from Sardinian Waters by Ribotyping and a New Rapid PCR Fingerprinting Method

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Received 8 December 1998/Accepted 1 February 1999

We investigated the usefulness of a novel PCR fingerprinting technique, based on the specific amplification of genomic regions, to differentiate 30 *Vibrio alginolyticus* strains isolated in Sardinian waters. The different profiles obtained were scanned and analyzed by a computer program in order to determine genetic relationships. The results were then compared with the patterns obtained by ribotyping with *Hin*dIII, *Kpn*I, and *Xba*I restriction enzymes. PCR fingerprinting could differentiate the strains analyzed into 12 different patterns, whereas ribotyping with *Xba*I, which produced the highest number of patterns, generated only 7 different profiles. This study revealed the superior discriminative power of the proposed technique for the differentiation of related *V. alginolyticus* strains and the potential use of PCR fingerprinting in epidemiological studies.

Members of the genus *Vibrio*, of the family *Vibrionaceae*, have acquired increasing importance because several are associated with human disease. Vibrios of medical importance include *Vibrio cholerae*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio mimicus* and, to a lesser extent, *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio hollisae*, and *Vibrio damsela* (1, 2, 5, 7, 8, 13, 18). Recent studies report several clinical infections caused by *Vibrio alginolyticus* (7, 13, 18). Bacteremic infections occurred only in patients with underlying diseases (13). In the Mediterranean area, *V. alginolyticus* has been the cause of several ear infections in humans (18). Both biotypes of halophilous vibrios, *Vibrio parahaemolyticus* and *V. alginolyticus*, were responsible for intestinal diseases among the inhabitants of the littoral localities of the Crimea (21). In the United States (i.e., the gulf states Alabama, Florida, Texas, and Louisiana), raw oyster consumption is an important cause of *Vibrio*-associated gastroenteritis among adults without underlying illnesses (13).

Isolation of *V. alginolyticus* from Mediterranean coastal water has been reported in Spain (18) and in Sicily, Italy (6), although neither study genotypically characterized the strains isolated. Several methods have been reported to characterize different *Vibrio* species (1, 2, 3, 11, 12, 16, 20); outer membrane protein profiling and phage typing were the first molecular methods to be used (4, 14). Ribotyping has been applied successfully to differentiate various bacterial species, including *V. vulnificus* (1, 2, 11, 22). Recently, random amplified polymorphic DNA (RAPD) has been applied to differentiate *Vibrio* species (1, 2, 11, 12, 15, 20). RAPD is based on low-stringency amplification by decreasing the temperature of annealing (11, 15). The patterns obtained can vary greatly in response to minimal changes in the amplification. To interpret the patterns obtained, some studies group in the same cluster bacteria that have only 77% similarity (2); moreover, the reproducibility of the experiment is very low (15).

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In order to characterize the *V. alginolyticus* strains isolated at the genotype level, we applied the ribotyping method and a new PCR fingerprinting method based on the amplification of conserved and specific internal sequences belonging to the IS*256* family, which includes IS*1245*, IS*1311*, IS*1081*, and IS*1395* (17).

MATERIALS AND METHODS

Bacterial strains. A total of 30 *V. alginolyticus* strains were isolated from samples of coastal water of northern Sardinia (Fig. 1). The samples were preenriched in alkaline peptone water (pH 8.6, 1% NaCl) at 37°C overnight, and yellow colonies were selected after 24 h of incubation on T.C.B.S. agar (Microbiol Diagnostici, Ca, Italy). The bacterial strains were presumptively identified by the API 20E system (BioMerieux, Marcy l'Etoile, France) (3). Ten were isolated from the Calich estuary of Alghero, three were from Porto Conte near Alghero, three were from the natural lake of Baratz, one was from a lake of Simbirizzi, seven were isolated in the seacoast of Tavolara island, and six were isolated from the seacoast waters of "La Maddalena" island. The strains were grown in marine broth 2216 (Microbiol Diagnostici) or Trypticase soy agar (Difco) supplemented with 0.5% (wt/vol) NaCl. Isolates were incubated at 25°C for 24 h.

DNA extraction and ribotyping. Chromosomal DNA extraction was performed as described by Wilson (25). The endonucleases *Hin*dIII, *Kpn*I, and *Xba*I (Amersham International, Amersham, United Kingdom) were used to digest the chromosomal DNA in order to determine the best differentiation of *V. alginolyticus* strains as previously observed by Hoi et al. (11) and Arias et al. (1). *Hin*dIII was reported by Hoi et al. (11) to produce the best results, whereas we found *Xba*I to be the best enzyme for differentiating *V. alginolyticus* strains. Two micrograms of chromosomal DNA was digested with *Xba*I as specified by the manufacturer. To separate the DNA restriction fragments obtained, the fragments were added to electrophoresis gels in 0.8% agarose (FMC Bioproducts, Rockland, Maine) in 1× TAE (0.01 M Tris-acetate, 0.1 mM EDTA; pH 8) buffer at 35 V for 12 h. For hybridization, DNA was transferred to a nylon membrane (Amersham) by the Southern method (23). Hybridizations were carried out at 65°C, and the blots were washed at 68°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; pH 7) and 0.1% sodium dodecyl sulfate. Probes were labeled by using the enhanced chemiluminescence gene detection system (Amersham) (20). The 1.8-kb *Apa*I clone (22) and λ DNA were used as probes.

PCR fingerprinting. Primers were designed to be complementary to conserved regions of the 5′ end of the IS*1245* and IS*1311* (10) and the 3′ end of the IS*1245* and *1311* belonging to the IS*256* family (10, 17), and they were synthesized by using a Gene-Assembler Plus (Pharmacia LKB) in our institute. Sequences of the primers were 5′-TCACTACCGAGAGGAACATC-3′ for p-95 and 5′-GGT GGAGGTGCCGTGCA-3′ for p-447 and the primers were used at a concentration of 0.5 μM. Amplification reactions were performed in 50 μl with 1 U (final volume) of *Taq* polymerase, 20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM deoxynucleoside triphosphate (GIBCO-BRL/Life Technology, Paisley, United Kingdom). Reaction mixtures were overlaid with one drop

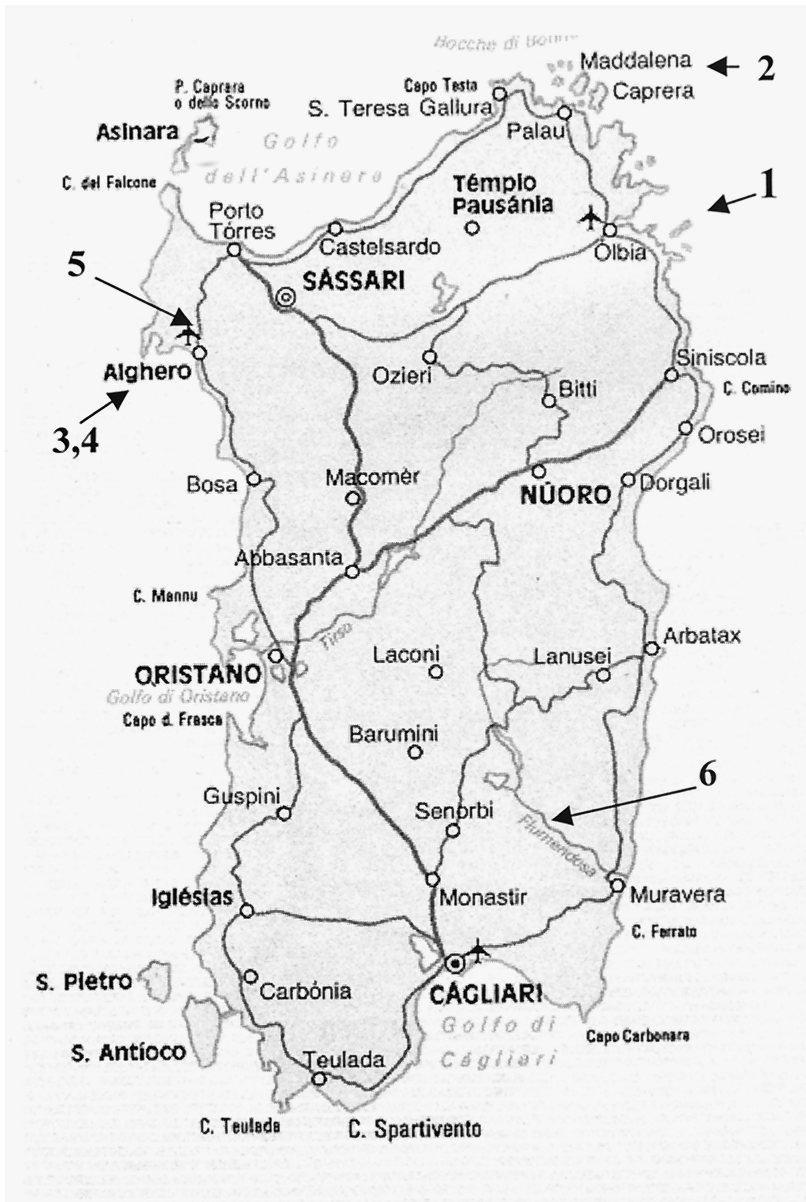

FIG. 1. Map of Sardinia. Arrows indicate the region of isolation of *V. alginolyticus* strains. 1, Tavolara; 2, La Maddalena; 3, Alghero; 4, Calich; 5, Baratz; 6, Simbirizzi.

of paraffin oil and then incubated for 2 min at 94°C, followed by 35 cycles at 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 20 min. The amplification products were visualized after electrophoresis at 90 V for 90 min in 2.5% Methaphore agarose gel (FMC Bioproducts), followed by staining of the gel with ethidium bromide. All DNA amplifications were performed in a Hybaid DNA thermal cycler instrument (Model TR3CM220; Omnigene).

Computer analysis of fingerprints. The patterns produced by the ribotyping method and PCR fingerprinting were evaluated with the Image Master software (Pharmacia). All bands produced were normalized by comparing the molecular weight markers (100-bp ladder) between different gels for PCR fingerprinting, and the molecular weights of the amplified bands were calculated by using the Image Master software. The dendrogram was generated by the UPGMA clustering method (deviation value of 0.025%).

RESULTS

Ribotyping. In Fig. 1 the regions where the 30 *V. alginolyticus* strains were isolated are shown. These strains were analyzed by ribotyping with the 3′ part of the 16S, the intergenic spacer, and the 5′ part of the 23S as a probe. The results are shown in Fig. 2 and Table 1. We used three restriction enzymes, *Hin*dIII, *Kpn*I, and *Xba*I, in order to obtain the best differentiation of the *V. alginolyticus* strains. *Xba*I produced seven different patterns of the 30 strains analyzed, whereas *Kpn*I and *Hin*dIII only produced four and three patterns, respectively (data not shown). The 10 strains isolated in the region of Calich, on the west coast of Sardinia, showed three different profiles (Table 1; Fig. 2, lanes A and D, and a profile similar to that shown in lane C). All three strains from Alghero generated ribotype pattern 6, as did one strain from La Maddalena and one strain from Tavolara (Fig. 2, lane C). The 13 strains from the La Maddalena and Tavolara islands generated three patterns of hybridization (types 1, 2, and 6) with the prevalence of pattern 1 (Table 1). In Fig. 2, lane G, is shown the pattern of strain L15

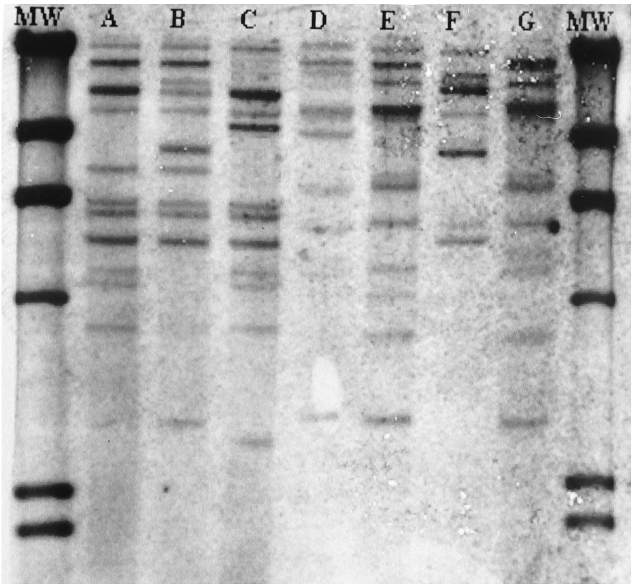

FIG. 2. Southern blot of chromosomal DNA showing representative examples of the different patterns obtained by ribotyping of the analyzed *V. alginolyticus* isolates. Lanes: A, L5 (Calich); B, L6 (Tavolara); C, L28 (Alghero); D, L29 (Calich); E, L7 (Baratz); F, L27 (Simbirizzi); G, L15 (La Maddalena); and MW, λ *Hin*dIII marker.

(type 1, isolated in La Maddalena sea waters); the other patterns found (type 2 and 6) are shown in Fig. 2, lanes B and C. These two islands are both on the Sardinian east coast and they are very close geographically, as can be seen in Fig. 1. The three strains isolated in the salty lake of Baratz showed the same ribotype (type 7; Fig. 2, lane E), whereas the Simbirizzi isolate generated a unique pattern (type 5; Fig. 2, lane F).

TABLE 1. Strains used in this study

Strain	Source (mo-yr)	Region of origin	No. of fingerprinting patterns found by: Ribotyping	IS-PCR
L13	Seawater (7-96)	Tavolara	1	1
L25	Seawater (7-96)	Tavolara	1	1
L16	Seawater (7-96)	Tavolara	1	2
L26	Seawater (7-96)	Tavolara	2	8
L6	Seawater (7-96)	Tavolara	2	10
L12	Seawater (7-96)	Tavolara	2	13
L11	Seawater (7-96)	Tavolara	6	12
L10	Seawater (6-98)	La Maddalena	6	12
L15	Seawater (1-96)	La Maddalena	1	1
L17	Seawater (8-96)	La Maddalena	1	2
L18	Seawater (8-96)	La Maddalena	1	2
L23	Seawater (8-96)	La Maddalena	1	2
L24	Seawater (8-96)	La Maddalena	1	2
L14	Seawater (7-96)	Alghero	6	2
L28	Seawater (6-98)	Alghero	6	2
L20	Seawater (6-98)	Alghero	6	2
L21	Lagoon (4-98)	Calich	6	2
L19	Lagoon (4-98)	Calich	4	3
L29	Lagoon (7-98)	Calich	3	4
L22	Lagoon (4-98)	Calich	3	5
L1	Lagoon (7-96)	Calich	4	6
L2	Lagoon (7-96)	Calich	4	6
L3	Lagoon (7-96)	Calich	4	6
L4	Lagoon (7-96)	Calich	4	6
L5	Lagoon (7-96)	Calich	4	6
L30	Lagoon (6-98)	Calich	4	6
L7	Salty lake (7-98)	Baratz	7	9
L8	Salty lake (7-98)	Baratz	7	9
L9	Salty lake (7-98)	Baratz	7	9
L27	Artificial lake (6-98)	Simbirizzi	5	11

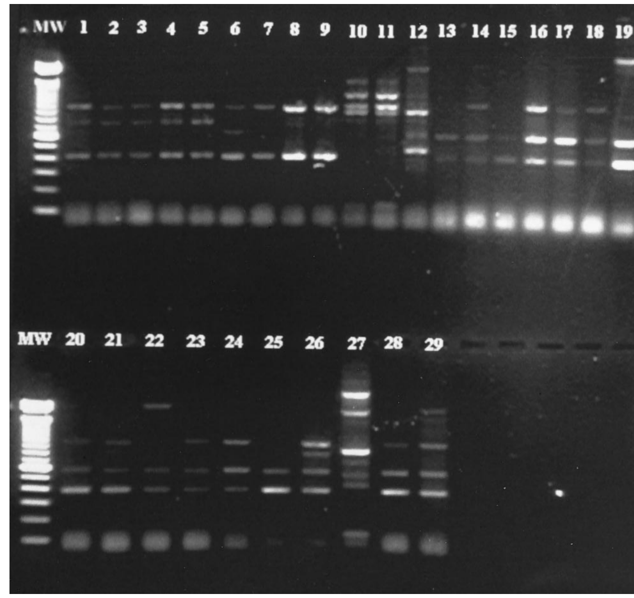

FIG. 3. Agarose gel electrophoresis of DNA amplifications of *V. alginolyticus* isolates obtained with the PCR fingerprinting method. Lanes: MW, 100-bp ladder (GIBCO/Life Science); 1, L1 (Calich); 2, L2 (Calich); 3, L3 (Calich); 4, L4 (Calich); 5, L5 (Calich); 6, L6 (Tavolara); 7, L7 (Baratz); 8, L8 (Baratz); 9, L9 (Baratz); 10, L10 (La Maddalena); 11, L11 (Tavolara); 12, L12 (Tavolara); 13, L13 (Tavolara); 14, L14 (Alghero); 15, L15 (La Maddalena); 16, L16 (Tavolara); 17, L17 (La Maddalena); 18, L18 (La Maddalena); 19, L19 (Calich); 20, L20 (Alghero); 21, L21 (Calich); 22, L22 (Calich); 23, L23 (La Maddalena); 24, L24 (La Maddalena); 25, L25 (Tavolara); 26, L26 (Tavolara); 27, L27 (Cagliari); 28, L28 (Alghero); and 29, L29 (Calich).

PCR fingerprinting. On the basis of conserved sequences among bacteria, we chose two primers complementary to conserved sequences of the transposase gene of IS*1311* and IS*1245* (10, 17). Representative examples of the results obtained are shown in Fig. 3, where we can observe the relatively large number of bands amplified from the different isolates (bands 2 to 6). In order to test the reproducibility of the technique, we amplified the chromosomal DNA of the different isolates after several subcultures; all of the strains tested showed the same amplification patterns in the different experiments performed (data not shown). In total, PCR fingerprinting of the 30 isolated *V. alginolyticus* strains produced 12 different profiles (Table 1). The patterns obtained were evaluated by the Image Master system analysis in order to determine the molecular weights of the bands, and the genetic distances among these strains were evaluated by the construction of a dendrogram, prepared with the Dendron software, on the basis of the patterns obtained (Fig. 4). The PCR fingerprinting clearly shows a higher level of differentiation than did the ribotyping. In particular, the three ribotyping families produced by the 13 strains from the La Maddalena and Tavolara islands were subdivided into six subfamilies by the PCR fingerprinting method (Table 1 and Fig. 4). The 10 strains isolated in the Calich region of Alghero, which generated three ribotype patterns, showed five different patterns of amplification, whereas the three strains from the lake of Baratz confirmed the results obtained with ribotyping, since they generated the same pattern of amplification (Table 1 and Fig. 3). The isolate from the artificial lake of Simbirizzi, L27, which showed a unique ribotyping pattern (type 5), also

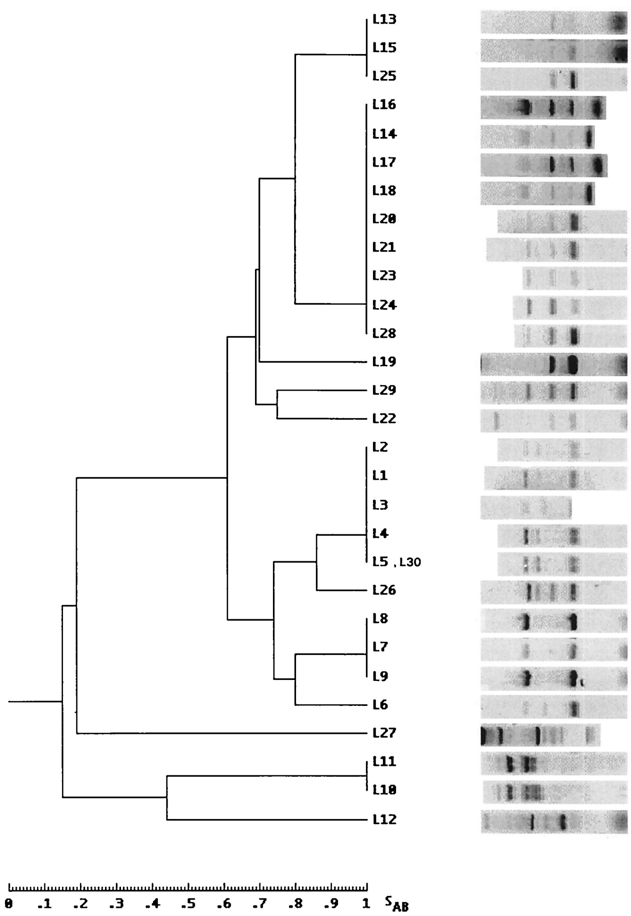

FIG. 4. Dendrogram with restriction fragment length polymorphism profiles illustrating the relationships among the 30 *V. alginolyticus* strains analyzed by PCR fingerprinting. Increasing similarity resulted in similarity index (S_{AB}) values ranging from 0 to 1.0.

produced a unique pattern with PCR fingerprinting (Table 1 and Fig. 4).

DISCUSSION

Several studies have been reported that use different strategies to fingerprint *V. vulnificus* strains (1, 2, 3, 11, 20). Among the molecular techniques used, ribotyping is the most diffuse (1, 2, 3, 11). Recently, the introduction of RAPD PCRs has shortened the time of typing (15). Enterobacterial repetitive consensus sequence PCR has been reported to produce polymorphic patterns when applied to different *Vibrio* species (9), but some studies claim that the complex patterns generated are produced by a mechanism similar to the RAPD method (9).

We report here the characterization of 30 *V. alginolyticus* strains isolated from Sardinian waters by ribotyping and a new PCR fingerprinting based on the amplification of specific insertion sequences. Ribotyping generates fingerprinting patterns reproducible over time, but it is not able to detect minimal changes in the bacterial genome, nor can it detect virulence characters which can change more rapidly than ribotype patterns within a bacterial species. In these cases we need a method that can detect such changes. In fact, although the patterns that we obtained from ribotyping were reproducible and easy to interpret, this method produced only seven different patterns from 30 different bacterial strains. The PCR method showed a greater ability to differentiate the strains analyzed, producing 12 different patterns. The strains isolated in the La Maddalena and Tavolara islands and those isolated in Alghero produced only three patterns by ribotype (types 1, 2, and 6; the latter type was found also in the Alghero and Calich isolates), whereas PCR fingerprinting could differentiate the same strains into six different families. The PCR fingerprinting method proposed is based on the use of primer sequences that are complementary to conserved IS sequences (10, 24). This PCR method was able to differentiate within ribotype families. It is possible that the amplified products obtained were not necessarily generated by annealing to genuine IS*256* sequences in the target genome, but they could be generated by amplification of other related insertion sequences. The observation that the PCR banding patterns obtained with these primer pairs were quite similar at annealing temperatures of both 50 and 60°C indicates that primer annealing is largely sequence specific rather than random. This PCR fingerprinting method is rapid and simple, and it may be a helpful tool for differentiating *V. alginolyticus* strains in epidemiological analyses, particularly in large studies and in urgent situations.

ACKNOWLEDGMENT

This work was supported by a grant of the Sardinian Region and the Provincia di Sassari.

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